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24 MAR 2003

3. Full name, address and postcode of the or of each applicant (underline all surnames)

1. Xzillion GmbH & Co. KG
Industriepark Hochst
Building G865, 65926 Frankfurt am Main
Germany (see attached sheet for further applicant)

Patents ADP number (if you know it)

8218158001

If the applicant is a corporate body, give the country/state of its incorporation

Germany

4. Title of the invention

MASS LABELS

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

PAGE WHITE & FARRER
54 Doughty Street,
London WC1N 2LS,
United Kingdom

Patents ADP number (if you know it)

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Country

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Christopher Michael Hill
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2. Proteome Sciences plc
Coveham House
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Patents ADP number (*if you know it*)

7671670001

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MASS LABELS

This invention relates to methods of labelling non-volatile analyte molecules particularly biomolecules with markers that assist in the purification, detection and analysis of the labelled biomolecule particularly with analysis by Electrospray Ionisation (ESI) and Matrix Assisted Laser Desorption Ionisation (MALDI) Mass Spectrometry. Specifically this invention relates to tertiary amino compounds linked to reactive functionalities that enable the labelling of amino groups in natural or synthetic biomolecules.

Mass spectrometry is a powerful tool in protein and peptide identification and structure analysis. Matrix-assisted laser desorption ionisation time of flight mass spectrometry (MALDI TOF MS) has become a robust tool for the identification of 2-D gel separated proteins by Peptide Mass Fingerprinting (Mann, Hojrup et al. 1993; Pappin, Höjrup et al. 1993; Yates, Speicher et al. 1993) while Electrospray Ionisation (ESI)-tandem mass spectrometry (MS/MS) coupled to high-performance liquid chromatography (HPLC) is emerging as the preferred technique for the analysis of digests of complex protein mixtures particularly in combination with novel peptide separation and isolation techniques, such as ICAT(Gygi, Rist et al. 1999; Han, Eng et al. 2001; Smolka, Zhou et al. 2001) and MudPIT(Washburn and Yates 2000; Washburn, Wolters et al. 2001; Wolters, Washburn et al. 2001; Washburn, Ulaszek et al. 2002).

Sequence information for peptides analysed by both ESI and MALDI can be obtained from their product-ion spectra after collision-induced dissociation (CID) of the protonated molecule with tandem mass spectrometric analysis. ESI-MS is however, particularly useful for peptide identification as it tends to produce better MS/MS spectra. This is due to a number of features of the ESI process. Ionisation by ESI imparts a very small amount of kinetic energy to the ions formed. In addition, atmospheric pressure ion sources rapidly equilibrate ion temperature through collisions. This allows, controlled acceleration of ions to impart precise amounts of kinetic energy. In addition, it is usual to couple ESI to tandem mass analysers with a dedicated collision cell which provides a controlled environment for low energy CID. Similarly, ion traps with their high operating pressure and selective activation of ions also provide controlled CID

conditions. In contrast, MALDI generates energetic ions in a near-vacuum, that typically fragment with a different mechanism to ESI. It is also unusual to have a collision cell in a MALDI instrument and the few instruments that do have a collision cell, typically operate at higher collision energies than the corresponding ESI instrumentation. Furthermore, ESI generates ions with higher charge states (Gaskell 1997) than MALDI, which typically produces singly charged ions (Karas, Gluckmann et al. 2000). This means that larger ions can be analysed in the functional range of the instrument and peptides with higher charge states generally give better CID spectra (Tang, Thibault et al. 1993) as more highly charged species give rise to multiple ion series providing redundant sequence information and sequence information from both termini of the peptide: the currently accepted model of peptide scission during CID requires protonation of the peptide backbone followed by nucleophilic attack of the carbonyl moiety of the protonated amide by the next N-terminal carbonyl residue in the peptide chain to form a relatively stable oxazolone leading to scission of the amide bond (Polce, Ren et al. 2000; Schlosser and Lehmann 2000) yielding two main fragment ion series of type b (containing N-terminal residues) and y (C-terminal residues), respectively. Higher charge states promote the formation of both ion classes ensuring a better chance of complete elucidation of peptide sequences. However, there are many pitfalls in the determination of peptide sequences by MS/MS. The b-series ions, for example, can lose the carbonyl moiety producing a further ion series, dubbed a type ions. A further problem, for CID of peptides is distinguishing isomeric (leucine and isoleucine) or isobaric (lysine and glutamine) amino acids.

It has been shown, however, that chemical modification of peptides can improve the results of MS/MS analyses. For instance, derivatisation of lysine allows glutamine to be distinguished (Bonetto, Bergman et al. 1997; Cardenas, van der Heeft et al. 1997). Sulphonic acid derivatives of the alpha-amino functionality of peptides have been shown to enhance fragmentation efficiency in MALDI-Ion Trap analysis of peptides with improved spectra for certain classes of peptides that typically give poor MS/MS spectra in the ion trap, such as peptides containing aspartic and glutamic acid (Keough, Lacey et al. 2001). The strongly acidic functionality facilitates protonation of the amide backbone of singly charged peptides in MALDI leading to increased fragmentation. Sulphonic acid derivatives of peptides have also been shown to

improve sensitivity of negative ion mode ESI-MS (Griffiths, Lindh et al. 1995). Aminonaphthalenesulphonic acid derivatives of peptides produce $[M-H]^-$ ions with good efficiency, which makes it possible to take advantage of the lower noise levels of the negative ion mode giving very high sensitivity.

Quaternary ammonium derivatisation reagents have been tested for a number of mass spectrometry techniques, including Fast Atom Bombardment (FAB) (Stults, Halualani et al. 1989), MALDI TOF (Bartlet-Jones, Jeffery et al. 1994) and ESI (Stults 1992) for the analysis of peptides. Typically sensitivity is not significantly enhanced by quaternary ammonium derivatisation in FAB, MALDI or ESI, with reduction in sensitivity reported in ESI applications (Stults 1992). However, fragmentation behaviour is modified by these sorts of tags: quaternary ammonium ion derivatised peptides show greater predominance of a- and d-series ions from peptides derivatised at the N-terminus and more abundant y-, v- and w-series for peptides derivatised on Lysine at the C-terminus (Roth, Huang et al. 1998).

As a further example, guanidination of lysine has been shown to enhance the sensitivity of the detection of lysine containing peptides in MALDI analyses (Bonetto, Bergman et al. 1997; Brancia, Oliver et al. 2000; Brancia, Butt et al. 2001). Similarly, N-succinimidyl-2-(3-pyridylacetate) (SPA) has been used for the derivatisation of peptides for ESI-MS/MS analysis (Sherman, Yates et al. 1995; Cardenas, van der Heeft et al. 1997). The SPA reagent has been shown to alter the CID fragmentation pathway, resulting in less complex product-ion spectra and the promotion of the formation of b-type ions. The reaction of SPA with peptides occurs selectively at the alpha and epsilon amino functions but not with the guanidino function of arginine.

Various other reagents for derivatising peptides have been also been developed. Halogenated compounds, particularly halogenated aromatic compounds are well known electrophores, i.e. they pick up thermal electrons very easily. A variety of derivatisation reagents based on fluorinated aromatic compounds (Bian N. et al., Rapid Commun Mass Spectrom 11(16): 1781-1784, "Detection via laser desorption and mass spectrometry of multiplex electrophore-labelled

albumin." 1997) have been developed for electron capture detection, which is a highly sensitive ionisation and detection process that can be used with negative ion mass spectrometry (Abdel-Baky S. & Giese R.W., Anal. Chem. 63(24):2986-2989, "Gas chromatography/electron capture negative-ion mass spectrometry at the zeptomole level." 1991).

Each type of derivitisation reagent that has been disclosed in the prior art has different benefits and limitations, which depend on the method of ionisation used and on the methods of mass analysis used (for a review see Roth et al., Mass Spectrometry Reviews 17:255-274, "Charge derivatisation of peptides for analysis by mass spectrometry", 1998). The mechanism by which sensitivity is enhanced may also be different for each type of group. Some derivitisation methods increase basicity and thus promote protonation and charge localisation, while other methods increase surface activity of the tagged peptides, which improves sensitivity in surface desorption techniques like Matrix Assisted Laser Desorption Ionisation (MALDI) and Fast Atom Bombardment (FAB). Negative ion mass spectrometry is often more sensitive because there is less background noise.

Most of the reactions discussed above have been performed on peptides rather than on whole proteins. In general, the kinetics of peptide derivatisation reactions are more favourable than the corresponding reactions of whole proteins, probably due to the reduced steric accessibility of reactive groups in large, folded polypeptides. Very little work has been reported on labelling of large polypeptides with any of the reagents above, which is an object of this invention.

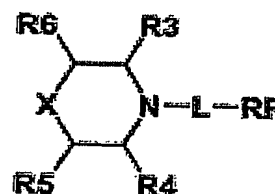
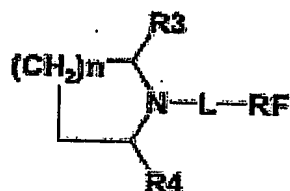
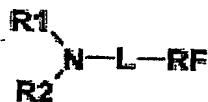
It is an object of this invention to provide methods and labels that can be used to produce protect amino groups in biomolecules, particularly proteins while retaining a functionality that can still protonate, which is advantageous for purification of labelled biomolecules and is also advantageous for analysis of the labelled biomolecules by mass spectrometry.

It is an object of this invention to provide compounds which have desirable features as mass labels and methods for the use of those compounds to provide improved mass spectra of associated analytes.

In a first aspect of this invention there is provided a Basic Mass Tag reagent with the following general structure:

Tertiary amino group—Linker-Reactive Functionality

where the Basic Mass Tag reagent is preferably selected from the group comprising:



where R₁, R₂, can be alkyl or aralkyl such as independently methyl, ethyl, propyl, iso-propyl, cyclohexyl, benzyl or substituted benzyl and preferably methyl, ethyl or iso-propyl;

R₃, R₄, R₅ and R₆ can be alkyl, such as independently, methyl, ethyl, propyl, iso-propyl or hydrogen and preferably methyl or hydrogen;

n can be comprised between 0 and 2 and preferably 2;

X can be independently an N-alkyl (where alkyl is referred as a methyl, ethyl, propyl, iso-propyl or cyclohexyl), S or O and preferably X is O;

and where RF, the reactive functionality, is preferably selected from the group comprising active esters of carbonic acids, alkenyl sulphones, haloalkanes, maleimides, isocyanates, isothiocyanates, ketones, aldehydes, sulphonyl-halides, carboxylic-halides, anhydride esters and alkenes and preferably active esters of carbonic acid such as independently N-hydroxysuccinimide esters, nitrophenyl esters, trichlorophenyl esters, pentafluorophenyl esters, hydroxybenzotriazole esters and hydroxyazabenzotriazole esters, (see Mizhiritskii and Shpermat 2002);

and where L, the linker, can be an alkylene linker such as independently $-(CH_2)_n-$ and substituted $-(CH_2)_n-$ (where n is comprised between 1 to 5), and phenylene such as $-(C_6H_4)-$ and preferably $-CH_2-$ and $-(CH_2)_3-$.

The substituents mentioned above are not particularly limited and may comprise any organic group and/or one or more atoms from any of groups IIIA, IVA, VA, VIA or VIIA of the Periodic Table, such as a B, Si, N, P, O, or S atom or a halogen atom (e.g. F, Cl, Br or I).

When the substituent comprises an organic group, the organic group preferably comprises a hydrocarbon group. The hydrocarbon group may comprise a straight chain, a branched chain or a cyclic group. Independently, the hydrocarbon group may comprise an aliphatic or an aromatic group. Also independently, the hydrocarbon group may comprise a saturated or unsaturated group.

When the hydrocarbon comprises an unsaturated group, it may comprise one or more alkene functionalities and/or one or more alkyne functionalities. When the hydrocarbon comprises a straight or branched chain group, it may comprise one or more primary, secondary and/or tertiary alkyl groups. When the hydrocarbon comprises a cyclic group it may comprise an aromatic ring, an aliphatic ring, a heterocyclic group, and/or fused ring derivatives of these groups. The cyclic group may thus comprise a benzene, naphthalene, anthracene, indene, fluorene, pyridine, quinoline, thiophene, benzothiophene, furan, benzofuran, pyrrole, indole, imidazole, thiazole, and/or an oxazole group, as well as regioisomers of the above groups.

The number of carbon atoms in the hydrocarbon group is not especially limited, but preferably the hydrocarbon group comprises from 1-40 C atoms. The hydrocarbon group may thus be a lower hydrocarbon (1-6 C atoms) or a higher hydrocarbon (7 C atoms or more, e.g. 7-40 C atoms). The number of atoms in the ring of the cyclic group is not especially limited, but preferably the ring of the cyclic group comprises from 3-10 atoms, such as 3, 4, 5, 6 or 7 atoms.

The groups comprising heteroatoms described above, as well as any of the other groups defined above, may comprise one or more heteroatoms from any of groups IIIA, IVA, VA, VIA or VIIA of the Periodic Table, such as a B, Si, N, P, O, or S atom or a halogen atom (e.g. F, Cl, Br or I). Thus the substituent may comprise one or more of any of the common functional groups in organic chemistry, such as hydroxy groups, carboxylic acid groups, ester groups, ether groups, aldehyde groups, ketone groups, amine groups, amide groups, imine groups, thiol groups, thioether groups, sulphate groups, sulphonic acid groups, and phosphate groups etc. The substituent may also comprise derivatives of these groups, such as carboxylic acid anhydrides and carboxylic acid halides.

In addition, any substituent may comprise a combination of two or more of the substituents and/or functional groups defined above.

In a preferred embodiment of the first aspect of this invention the Basic Mass Tag (BMT) reagent has a characteristic isotope abundance distribution. In one such embodiment, the BMT is substituted with one or more halogen atoms or with atoms of stable isotopes such as deuterium, ^{13}C and/or ^{15}N . In a second embodiment, the BMT reagent comprises a mixture of two or more isotopes of the same compound, such that the reagent imparts a characteristic distribution of isotopes to a population of labelled ions.

The invention also provides an array of mass tags for labelling one or more molecules to be characterised by mass spectrometry, which array comprises two or more mass tags as defined above. In one embodiment, every mass tag in the array has the same chemical structure, and each mass tag in the array is an isotopomer of the other mass tags in the array such that each mass tag in the array has a different mass. These arrays of tags are useful for investigating a plurality of analytes simultaneously.

In a second typical aspect of this invention there is provided a method of analysing a polypeptide comprising the steps of:

1. labelling free amino functionalities in the polypeptide with a reactive, tertiary-amino group containing reagent;
2. cleaving the polypeptide with a sequence specific cleavage reagent;
3. labelling the free amino groups generated in the peptides by the cleavage reagent with a second amino-reactive reagent; and
4. analysing the cleavage peptides by mass spectrometry.

In preferred embodiments of this aspect of the invention, the labelled peptides are separated by application of one or more analytical separation techniques prior to analysis by mass spectrometry. In more preferred, embodiments the labelled peptides generated by the cleavage reagent are purified by analytical separation of the peptides using strong cation exchange (SCX) chromatography. In still more preferred embodiments, the labelled peptides are purified by analytical separation of the peptides using SCX as part of a multi-dimensional analytical separation in which a number of different separation techniques are applied in sequence to the labelled peptides.

The invention also provides a kit comprising the Basic Mass Tags of this invention and a cation exchange resin for purification of labelled analyte molecules.

In preferred embodiments the kit may additionally comprise reaction buffers for the coupling of the tag to analyte molecules, buffers for washing of the cation exchange resin after the reaction buffer has been loaded onto the resin and buffers for elution of the labelled peptides from the resin after the unreacted tags have been washed away. A preferred wash buffer comprises water and acetonitrile at a low pH while a preferred elution buffer comprises ammonium acetate. In preferred embodiments the reaction buffer comprises borate, urea and thiourea. Preferably, the urea and thiourea are present at a combined concentration of at least 1M.

The invention will now be described in more detail by way of example only, with reference to the figures, in which:

Figure 1 shows the molecular structures of tags for which synthetic protocols are provided in the 'Examples' section below; and

Figure 2 shows retention times of singly labelled and unlabelled peptides plotted against each other.

Tertiary Amino Groups

The Basic Mass Tag reagents of the first aspect of this invention comprise a tertiary amino group linked to an amine-reactive functionality. The purpose of these reagents is to protect primary amino groups in natural or synthetic biomolecules, particularly proteins, while retaining a functionality that can still protonate, which is advantageous for purification of labelled biomolecules and is also advantageous for analysis of the labelled biomolecules by mass spectrometry, particularly for ionisation techniques that primarily rely on protonation for ionisation of analytes such as Electrospray Ionisation and MALDI TOF. Tertiary amines readily protonate but do not readily participate in nucleophilic attack on electrophiles. The substituents to the nitrogen in a tertiary amino group can be easily modified to allow the accessibility of the nitrogen to be altered and to alter the proton affinity of the nitrogen.

Tertiary amines are advantageous for the labelling of peptides as they retain a 'mobile' proton which facilitates sequencing of peptides by collision induced dissociation of the peptides (Schlosser and Lehmann 2000; Wysocki, Tsaprailis et al. 2000). Thus the tags of this invention provide a protecting group that allows the labelled peptide to behave in a manner similar to the unlabelled species when analysed by mass spectrometry.

Similarly, with the preferred compounds of this invention only a very small change in retention times is noticed when comparing the chromatographic separation of the labelled peptides and the unlabelled peptides.

For the purposes of this invention methyl groups are sufficient to protect the nitrogen when it is connected to a reactive functionality via a linear alkyl chain. N,N-Dimethyl glycine, for example

is a good reagent for the preparation of active esters. The N-hydroxysuccinimide ester of this compound is a preferred tag provided by this invention. More hindered substituents are also applicable but very large substituents are not preferred as these will interfere with the chromatographic properties of labelled biomolecules.

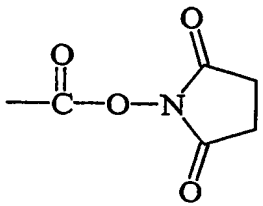
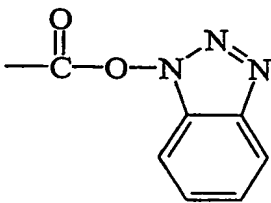
Reactive tags comprising tertiary amino functionalities

In the first aspect of this invention reactive tertiary amino group containing molecules are provided. Such reagents can be prepared from a number of commercially available intermediates. A number of compounds in which tertiary amino groups are linked to carboxylic acid functionalities, are commercially available (see 'Examples' section below). The carboxylic acid functionality may be readily converted to an active ester or acid chloride by conventional chemical methods (see for example Solomons, "Organic Chemistry", Fifth Edition published by Wiley). Preferred active esters include N-hydroxysuccinimide (NHS) esters and pentafluorophenyl esters.

Reactive Functionalities

A variety of other reactive functionalities may be appropriate to prepare reactive tertiary amino-containing reagents for use with this invention. Table 1 below lists some reactive functionalities that may be incorporated into the reagents of this invention. These reactive functionalities may be reacted with amino groups in natural or synthetic biomolecules, particularly in peptides and polypeptides. Reaction of the reactive functionalities with the nucleophilic functionalities shown generates a covalent linkage between the two entities. This covalent linkage is shown in the third column of the table. For applications involving synthetic oligonucleotides, primary amines are often introduced during the synthesis at the termini of the molecules to permit labelling. Any of the functionalities listed below could be introduced into the compounds of this invention to permit the mass markers to be attached to a molecule of interest. A reactive functionality can be used to introduce a further linker groups with a further reactive functionality if that is desired. Table 1 is not intended to be exhaustive and the present invention is not limited to the use of only the listed functionalities.

Table 1

| Nucleophilic Functionality | Reactive Functionality | Resultant Linking Group |
|----------------------------|--|--|
| -NH ₂ | -SO ₂ -CH=CR ₂ | -N(CR ₂ -CH ₂ -SO ₂ -) ₂ or -NH-CR ₂ -CH ₂ -SO ₂ - |
| -NH ₂ | -CO-X | -CO-NH- |
| -NH ₂ | -CO-O-CO- | -CO-NH- |
| -NH ₂ |  | -CO-NH- |
| -NH ₂ |  | -CO-NH- |
| -NH ₂ | -NCO | -NH-CO-NH- |
| -NH ₂ | -NCS | -NH-CS-NH- |
| -NH ₂ | -CHO | -CH ₂ -NH- |
| -NH ₂ | -SO ₂ -X | -SO ₂ -NH- |
| -NH ₂ | CH ₂ =CH- | -NH-CH ₂ -CH ₂ - |

It should be noted that in applications involving labelling oligonucleotides with the mass markers of this invention, some of the reactive functionalities above or their resultant linking groups might have to be protected prior to introduction into an oligonucleotide synthesiser. Preferably unprotected ester, thioether and thioesters, amine and amide bonds are to be avoided, as these are not usually stable in an oligonucleotide synthesiser. A wide variety of protective groups is known in the art which can be used to protect linkages from unwanted side reactions. Alternatively, amine functionalised oligonucleotides can be prepared using standard methods known in the art and these can be labelled with amine reactive reagents such as NHS-esters.

Basic Mass Tags with characteristic isotope abundance distributions

While peptides have characteristic isotope abundance distributions, it is often worthwhile to modify the isotope abundance distributions of peptides to allow specific features to be identified. One way of identifying cysteine containing peptides is to tag the cysteines with a label that gives the peptides a characteristic isotope distribution. A number of labels and tagging procedures have been developed for this purpose (Sechi and Chait 1998; Adamczyk, Gebler et al. 1999; Goodlett, Bruce et al. 2000; Sechi 2002).

In a typical analysis of a complex peptide mixture, the peptides are separated by HPLC, for example, and the eluting material is sprayed directly into a mass spectrometer through an ESI interface for analysis. Usually peptides are identified by MS/MS in an appropriate instrument. Since identification of peptides by MS/MS is moderately time-consuming, the speed of this type of analysis determines the overall throughput of the instrument for identification of peptides in samples. In instruments such as Quadrupole/Time-of-Flight machines in which MS-mode acquisition of spectra is comparatively rapid, the MS-mode can be used to identify ions as they are generated to determine which species to analysis by MS/MS. However, with unmodified peptides, it is sometimes difficult to distinguish between peptide ions and non-peptide contaminants in the MS-mode. This means that ions that are not peptides are often selected for MS/MS analysis, reducing the overall throughput of the instrument for peptide identification. To overcome this problem, this invention provides tags for the labelling of amino groups in polypeptides and peptides that alter the isotope abundance distribution of polypeptides and peptides. Since most peptides or polypeptides have at least one amino group this provides a way of tagging polypeptides or peptides to allow their ions to be distinguished from other ions present in the MS-mode by the modified isotope abundance distributions of the labelled peptides. This is advantageous as it ensures that time is not wasted analysing ions by CID that are not peptides. Thus in preferred embodiments of the first aspect of this invention, BMT reagents with characteristic isotope distributions are provided. When used to label a peptide or polypeptide these tags will modify the isotope distribution of the labelled species. In one embodiment, a characteristic isotope distribution can be imparted to tags of this invention by incorporation of one or more halogen atoms into the tag, generally substituting for hydrogen atoms. Both

bromine(Steen and Mann 2002) and chlorine(Goodlett, Bruce et al. 2000) have been used for this purpose in the prior art and would be appropriate. These atoms will increase the intensity of higher mass isotope peaks when compared to corresponding hydrogenated species. Similarly, fluorine will alter the isotope distribution of a tagged species as it is known to comprise almost exclusively a single isotope, so higher mass isotopes will be of reduced intensity when compared with the corresponding hydrogenated species. Halogen atoms will also introduce a mass defect(Steen and Mann 2002) into the tags which provides a further means for identifying tagged peptides.

It has been previously shown that a reagent for the labelling of cysteine that imparts a characteristic isotope distribution can be prepared from a mixture of compounds comprising natural and isotope enriched species of the same molecule(Adamczyk, Gebler et al. 1999). In a further embodiment, a tag reagent comprising a mixture of BMT reagent isotopes is provided. It is possible to obtain isotope enriched compounds for the synthesis of tags enriched for particular atomic species. For the purpose of illustration consider the tags provided in the 'Examples' section below, in which bromoacetic acid is used as one of the building blocks of a number of reagents. It is possible to obtain 99% ^{13}C -enriched bromoacetic acid ($\text{Br}^{13}\text{CH}_2^{13}\text{COOH}$) from Aldrich (Sigma-Aldrich Chemie GmbH, Eschenstrasse 5, 82024, Taufkirchen, Germany). It would, therefore, be trivial to introduce this reagent into the synthetic procedures described below. This tag would have a characteristic isotope abundance distribution itself but it would also be possible to mix this reagent with the corresponding reagent synthesized from bromoacetic acid comprising a natural isotope abundance. Different ratios of the two tags would allow tags with controlled isotope abundance distributions to be generated.

Further labelling of cleavage peptides generated from BMT labelled proteins

In the second aspect of this invention, a BMT labelled polypeptide is cleaved with a sequence specific cleavage reagent and the cleavage peptides are analysed by mass spectrometry. In preferred embodiments of this aspect of the invention, the cleavage peptides are labelled further on the amino groups exposed by the cleavage of the peptides. Since the labelling of the polypeptide will have blocked all other free amino groups, only the newly exposed amino groups

at the N-termini of the cleavage peptides will be available. Furthermore, the N-terminal peptide of the parent polypeptide will have no free amino group after cleavage as this will have been blocked with the BMT reagent of this invention.

This provides a means of distinguishing the N-terminal fragment of the parent polypeptide from the non-N-terminal peptides. In one embodiment, the non-N-terminal fragments can be labelled by biotinylation with an amine-reactive biotin reagent such as N-hydroxysuccinimide biotin, which is commercially available (Pierce UK Ltd, Chester, UK or Sigma-Aldrich, Poole, Dorset, UK), and captured onto an avidinated support allowing the N-terminal peptide to be isolated. Alternatively, the alpha-amino groups of the non-N-terminal peptides can be reacted with a primary-amine reactive solid support leaving the N-terminal peptide in solution.

In an alternative embodiment, the BMT labelled cleavage peptides can be labelled with an amine-reactive biotin reagent to allow the peptides to be captured onto an avidinated support for conditioning of the peptides prior to analysis by mass spectrometry. Conditioning of the peptides means washing of the peptides with a volatile buffer to remove metals ions, detergent and other reagents that were used in the isolation and labelling of the parent polypeptide.

In a further embodiment, the cleavage peptides can be labelled with a further tag such as those disclosed in WO 01/68664 and PCT/GB02/04240, which disclose organic molecule mass markers that are analysed by MS/MS. These applications disclose two component mass markers connected by a collision cleavable group. Sets of tags are synthesised where the sum of the masses of the two components produces markers with the same overall mass. The mass markers may be analysed after cleavage from their analyte or may be detected while attached to the analyte. In the context of this invention the mass markers are detected while attached to the peptide that they are identifying. Selection of the mass of the mass marker with its associated peptide by the first mass analyser of a tandem instrument allows the marked peptides to be abstracted from the background. Collision of the markers in the second stage of the instrument separates the two components of the tag from each other. Only one of these components is detected in the third mass analyser. This allows confirmation that the peak selected in the first

analyser is a mass marked peptide. The whole process greatly enhances the signal to noise ratio of the analysis and improves sensitivity. This mass marker design also compresses the mass range over which an array of mass markers is spread. Moreover, it allows the design of markers, which are chemically identical, have the same mass but which are still resolvable by mass spectrometry. This is essential for analytical techniques such as Liquid Chromatography Mass Spectrometry (LC-MS) where the effect of different markers on the mobility of different samples of peptides must be minimised so that corresponding peptides from each sample elute together into the mass spectrometer, allowing the ratios of the corresponding peptides to be determined. These markers are thus most preferred for the purposes of this invention because of the use of high selectivity detection and the closely related structures of these markers. Other markers may also be applicable, though.

Proteins contain various nucleophilic functionalities, besides amino groups, that can be labelled using reagents that are reactive to these functionalities. Proteins typically contain thiol, amino, hydroxyl and imidazole groups. These may all be labelled with appropriate reagents if desired. In preferred embodiments of this invention, thiol groups are labelled prior to labelling of free amino groups. Numerous methods for selective labelling of thiols are known in the art, although preferred reagents include iodoacetamide, vinylpyridine, phenyl vinyl sulphone and maleimide compounds.

Analysis of labelled biomolecules

In the second aspect of this invention, a method of analysing polypeptides is provided. In this method the biomolecules are covalently labelled with a reactive tertiary amino group containing compound.

A polypeptide or peptide or mixtures of polypeptides or peptides can be isolated for analysis by any of the conventional means such as electrophoresis, chromatography or affinity chromatography. For the purposes of mass spectrometry, particularly MALDI TOF mass spectrometry, it is preferred that polypeptides or proteins are not contaminated with salts or detergents, particularly metal salts. Various techniques for 'desalting' a polypeptide or peptide

mixture are known in the art such as gel filtration, dialysis or the use of hydrophobic resins. A particularly convenient and simple method for de-salting peptides involves aspiration of a small quantity of a solution of the peptide or polypeptide mixture in a pipette tip incorporating C18 packing materials. Hydrophobic material, such as salts and detergents have only a weak affinity for the C18 derivatives and are easily washed through while peptides adhere to the C18 matrix. The captured peptide material can be subsequently eluted with an appropriate volatile buffer for analysis. This sort of 'sample conditioning' substantially improves the detection sensitivity of the analysis of the peptides. Pipette tips pre-packaged with appropriate resins and instructions for their use are commercially available from Millipore (Bedford, MA, USA) under the trademark 'Zip Tip'. Desalting procedures may take place after labelling of the analyte to remove unreacted tags, if preferred.

The inventors have also observed that peptides labelled with the tags of this invention have a higher affinity for cation exchange resins, particularly strong cation exchange resins, than the tags alone. This allows peptides labelled with the tags of this invention to be purified from unreacted tags by incubation of the reaction mixture with a cation exchange resin. The labelled peptides adhere to the resin, while the unreacted tag, detergents and chaotropes can be washed away with an acidic aqueous solvent. The labelled peptides can then be eluted with an appropriate buffer. For the purposes of subsequent mass spectrometric analysis, elution with a volatile buffer like ammonium acetate, is effective to recover the peptides in a form that is compatible with the mass spectrometer. Accordingly, it is envisaged that pipette tips, spin columns and cartridges packed with a cation exchange resin will be useful tools for the preparation of labelled samples to allow facile clean-up of the labelled peptides prior to analysis.

Analytical Separation of Tagged Peptides

In preferred embodiments of the second aspect of this invention the tagged peptides generated by cleavage of BMT labelled proteins are subjected to analytical separation prior to analysis by mass spectrometry. Most analytical separation techniques that can be applied to peptides will be applicable with this invention, such as Capillary Electrophoresis (Moini 2002), High Performance Liquid Chromatography (Morand, Burt et al. 2001), Capillary Iso-electric Focusing

(Tang, Harrata et al. 1997; Shen, Berger et al. 2000), Ion Exchange Chromatography and Size Exclusion Chromatography(Liu, Lin et al. 2002).

In more preferred embodiments multidimensional chromatography(Washburn, Wolters et al. 2001; Wolters, Washburn et al. 2001; Liu, Lin et al. 2002) is applied to the tagged peptides generated by cleavage of BMT labelled proteins.

Mass Spectrometry of Tagged Peptides

The essential features of a mass spectrometer are as follows:

Inlet System -> Ion Source -> Mass Analyser -> Ion Detector -> Data Capture System

There are various inlet systems, ion sources and mass analysers that can be used for the purposes of analysing large biomolecules but in the context of this invention the ion source is preferably and Electrospray Ionisation (ESI) source or a Matrix Assisted Laser Desorption Ionisation (MALDI) source. ESI can be coupled to most types of mass analysers including Ion Traps, Quadrupole and Triple Quadrupole instruments, orthogonal axis Time-of-Flight and Quadrupole/Time-of-flight mass analysers. Similarly, a variety of mass analysers, ion detectors and data capture systems may be used with MALDI although some mass spectrometer geometries are not commercially produced. Time-of-flight mass analysers are typically used with MALDI as well as Fourier Transform Ion Cyclotron Resonance mass analysers and Quadrupole/Time-of-flight mass analysers. In principle ion traps and sector instruments can also be used with MALDI but generally these are not widely available.

Electrospray Ionisation

Electrospray ionisation requires that a dilute solution of the analyte molecule is 'atomised' into the spectrometer, i.e. injected as a fine spray. The solution is typically sprayed from the tip of a charged needle in a stream of dry nitrogen and an electrostatic field. The mechanism of ionisation is not fully understood but is thought to work broadly as follows. In a stream of nitrogen the solvent is evaporated. With a small droplet, this results in concentration of the

analyte molecule. Given that most biomolecules have a net charge this increases the electrostatic repulsion of the dissolved molecule. As evaporation continues this repulsion ultimately becomes greater than the surface tension of the droplet and the droplet disintegrates into smaller droplets. This process is sometimes referred to as a 'Coulombic explosion'. The electrostatic field helps to further overcome the surface tension of the droplets and assists in the spraying process. The evaporation continues from the smaller droplets which, in turn, explode iteratively until essentially the biomolecules are in the vapour phase, as is all the solvent. This technique is of particular importance in the use of mass labels in that the technique imparts a relatively small amount of energy to ions in the ionisation process and the energy distribution within a population tends to fall in a narrower range when compared with other techniques. The ions are accelerated out of the ionisation chamber by the use of electric fields that are set up by appropriately positioned electrodes. The polarity of the fields may be altered to extract either negative or positive ions. The potential difference between these electrodes determines whether positive or negative ions pass into the mass analyser and also the kinetic energy with which these ions enter the mass spectrometer. This is of significance when considering fragmentation of ions in the mass spectrometer. The more energy imparted to a population of ions the more likely it is that fragmentation will occur through collision of analyte molecules with the bath gas present in the source. By adjusting the electric field used to accelerate ions from the ionisation chamber it is possible to control the fragmentation of ions. This is advantageous when fragmentation of ions is to be used as a means of removing tags from a labelled biomolecule.

Matrix Assisted Laser Desorption Ionisation (MALDI)

MALDI requires that the biomolecule solution be embedded in a large molar excess of a photo-excitabile 'matrix'. The application of laser light of the appropriate frequency results in the excitation of the matrix which in turn leads to rapid evaporation of the matrix along with its entrapped biomolecule. Proton transfer from the acidic matrix to the biomolecule gives rise to protonated forms of the biomolecule which can be detected by positive ion mass spectrometry, particularly by Time-Of-Flight (TOF) mass spectrometry. Negative ion mass spectrometry is also possible by MALDI TOF. This technique imparts a significant quantity of translational energy to ions, but tends not to induce excessive fragmentation despite this. Accelerating

voltages can again be used to control fragmentation with this technique though. This technique is highly favoured for the determination of peptide mass fingerprints due to its large mass range, due to the prevalence of singly charged ions in its spectra and due to the ability to analyse multiple peptides simultaneously.

The photo-excitabile matrix comprises a 'dye', i.e. a compound that strongly absorbs light of a particular frequency, and which preferably does not radiate that energy by fluorescence or phosphorescence but rather dissipates the energy thermally, i.e. through vibrational modes. It is the vibration of the matrix caused by laser excitation that results in rapid sublimation of the dye which simultaneously takes the embedded analyte into the gas phase.

Mass Analysers

Time-of-Flight Mass Analysers

As the name implies, Time-of-flight mass analysers measure the time it takes for ions to travel a predetermined distance under the influence of a predetermined potential difference. The time-of-flight measurement allows the mass-to-charge ratio of ions striking a detector to be calculated. These instruments measure the arrival of almost all of the ions in a sample and as a result can be quite sensitive although, selectivity with this technique is more difficult to achieve. This technique can also detect ions with high mass-to-charge ratios than can be typically measured in an ion trap or quadrupole mass spectrometer. TOF mass analysers are presently widely used with MALDI.

Ion Traps

Ion Trap mass analysers are related to the quadrupole mass analysers. The ion trap generally has a 3 electrode construction - a cylindrical electrode with 'cap' electrodes at each end forming a cavity. A sinusoidal radio frequency potential is applied to the cylindrical electrode while the cap electrodes are biased with DC or AC potentials. Ions injected into the cavity are constrained to a stable circular trajectory by the oscillating electric field of the cylindrical electrode. However, for a given amplitude of the oscillating potential, certain ions will have an unstable trajectory and

will be ejected from the trap. A sample of ions injected into the trap can be sequentially ejected from the trap according to their mass/charge ratio by altering the oscillating radio frequency potential. The ejected ions can then be detected allowing a mass spectrum to be produced.

Ion traps are generally operated with a small quantity of a 'bath gas', such as helium, present in the ion trap cavity. This increases both the resolution and the sensitivity of the device as the ions entering the trap are essentially cooled to the ambient temperature of the bath gas through collision with the bath gas. Collisions both increase ionisation when a sample is introduced into the trap and dampen the amplitude and velocity of ion trajectories keeping them nearer the centre of the trap. This means that when the oscillating potential is changed, ions whose trajectories become unstable gain energy more rapidly, relative to the damped circulating ions and exit the trap in a tighter bunch giving a narrower larger peaks.

Ion traps can mimic tandem mass spectrometer geometries, in fact they can mimic multiple mass spectrometer geometries allowing complex analyses of trapped ions. A single mass species from a sample can be retained in a trap, i.e. all other species can be ejected and then the retained species can be carefully excited by super-imposing a second oscillating frequency on the first. The excited ions will then collide with the bath gas and will fragment if sufficiently excited. The fragments can then be analysed further. It is possible to retain a fragment ion for further analysis by ejecting other ions and then exciting the fragment ion to fragment. This process can be repeated for as long as sufficient sample exists to permit further analysis. It should be noted that these instruments generally retain a high proportion of fragment ions after induced fragmentation. These instruments and FTICR mass spectrometers (discussed below) represent a form of temporally resolved tandem mass spectrometry rather than spatially resolved tandem mass spectrometry which is found in linear mass spectrometers.

Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR MS)

FTICR mass spectrometry has similar features to ion traps in that a sample of ions is retained within a cavity but in FTICR MS the ions are trapped in a high vacuum chamber by crossed electric and magnetic fields. The electric field is generated by a pair of plate electrodes that form

two sides of a box. The box is contained in the field of a superconducting magnet which in conjunction with the two plates, the trapping plates, constrain injected ions to a circular trajectory between the trapping plates, perpendicular to the applied magnetic field. The ions are excited to larger orbits by applying a radio-frequency pulse to two 'transmitter plates' which form two further opposing sides of the box. The cycloidal motion of the ions generate corresponding electric fields in the remaining two opposing sides of the box which comprise the 'receiver plates'. The excitation pulses excite ions to larger orbits which decay as the coherent motions of the ions is lost through collisions. The corresponding signals detected by the receiver plates are converted to a mass spectrum by Fourier Transform (FT) analysis.

For induced fragmentation experiments these instruments can perform in a similar manner to an ion trap - all ions except a single species of interest can be ejected from the trap. A collision gas can be introduced into the trap and fragmentation can be induced. The fragment ions can be subsequently analysed. Generally fragmentation products and bath gas combine to give poor resolution if analysed by FT analysis of signals detected by the 'receiver plates', however the fragment ions can be ejected from the cavity and analysed in a tandem configuration with a quadrupole, for example.

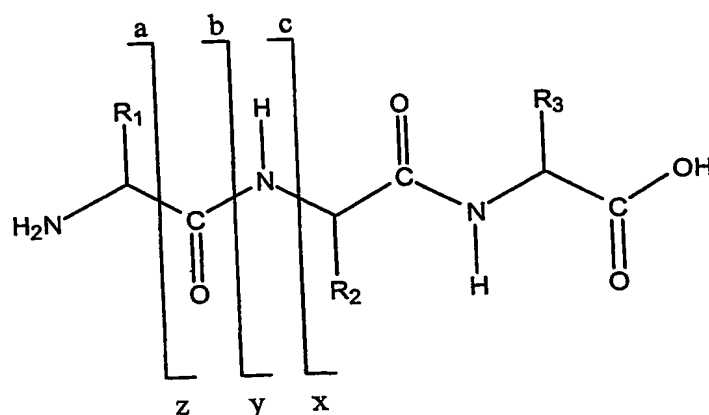
Identification of peptides by collision induced dissociation

In the second aspect of this invention the tagged peptides generated by cleavage of BMT labelled polypeptides are analysed by mass spectrometry. This analysis may include identification of the peptides by mass spectrometry. Various mass analyser geometries may be used to fragment peptides and to determine the mass of the fragments such as ion traps, triple quadrupoles, quadrupole/time-of-flight, time-of-flight/time-of-flight and serial sector instruments.

MS/MS and MSⁿ analysis of peptides:

Tandem mass spectrometers allow ions with a pre-determined mass-to-charge ratio to be selected and fragmented by collision induced dissociation (CID). The fragments can then be detected providing structural information about the selected ion. When peptides are analysed by CID in a tandem mass spectrometer, characteristic cleavage patterns are observed, which allow the

sequence of the peptide to be determined. Natural peptides typically fragment randomly at the amide bonds of the peptide backbone to give series of ions that are characteristic of the peptide. CID fragment series are denoted a_n , b_n , c_n , etc. for cleavage at the n^{th} peptide bond where the charge of the ion is retained on the N-terminal fragment of the ion. Similarly, fragment series are denoted x_n , y_n , z_n , etc. where the charge is retained on the C-terminal fragment of the ion.



Trypsin, Lys-C and thrombin are favoured cleavage agents for tandem mass spectrometry as they produce peptides with basic groups at both ends of the molecule, i.e. the alpha-amino group at the N-terminus and lysine or arginine side-chains at the C-terminus. This favours the formation of doubly charged ions, in which the charged centres are at opposite termini of the molecule. These doubly charged ions produce both C-terminal and N-terminal ion series after CID. This assists in determining the sequence of the peptide. Generally speaking only one or two of the possible ion series are observed in the CID spectra of a given peptide. In low-energy collisions typical of quadrupole based instruments the b-series of N-terminal fragments or the y-series of C-terminal fragments predominate. If doubly charged ions are analysed then both series are often detected. In general, the y-series ions predominate over the b-series.

In general peptides fragment via a mechanism that involves protonation of the amide backbone followed by intramolecular nucleophilic attack leading to the formation of a 5-membered oxazolone structure and cleavage of the amide linkage that was protonated (Polce, Ren et al.

2000; Schlosser and Lehmann 2000). This mechanism requires a carbonyl group from an amide bond adjacent to a protonated amide on the N-terminal side of the protonated amide to carry out the nucleophilic attack. A charged oxazolonium ion gives rise to b-series ions, while proton transfer from the N-terminal fragment to the C-terminal fragment gives rise to y-series ions. This requirement for an appropriately located carbonyl group does not account for cleavage at amide bonds adjacent to the N-terminal amino acid, when the N-terminus is not protected and, in general, b-series ions are not seen for the amide between the N-terminal and second amino acid in a peptide. However, peptides with acetylated N-termini do meet the structural requirements of this mechanism and fragmentation can take place at the amide bond immediately after the first amino acid by this mechanism. Peptides labelled on lysine or at an alpha-amino group with active ester tags of this invention should not undergo significant cleavage at the tag peptide during low energy CID analysis if there is no appropriately located carbonyl group in the tag molecule. This means that CID spectra for labelled peptides should be similar to the spectra for unlabeled peptides although the spectra will show mass shifts corresponding to multiples of the mass of the tag. This can be readily compensated for in the analysis of such CID spectra.

Similarly, peptides can be identified in single Time-of-Flight (TOF) mass analysers by a process referred to as Post-Source Decay (PSD) (Kaufmann, Chaurand et al. 1996). This is particularly effective in TOF mass analysers equipped with Delayed Extraction ion optics. MALDI tends to impart a high level of kinetic energy to analyte molecules that can cause fragmentation of the ions which can be used to deduce the sequence of the parent peptide ion.

EXAMPLES

The following examples illustrate some of the features and possible embodiments of the invention but other embodiments are envisaged.

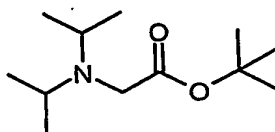
Syntheses of Basic Mass Tags

All reagents were obtained from Aldrich (Sigma-Aldrich Chemie GmbH, Eschenstrasse 5, 82024, Taufkirchen, Germany).

Basic Mass Tags were synthesised by activation of the corresponding carboxylic acid forms of the compounds to give the N-hydroxysuccinimide esters by standard methods. The reagents N,N-dimethylglycine, N,N-3-aminobutyric acid are commercially available.

DIG, PAA, MAA and DMA were prepared by reaction of Diisopropylamine, Piperidine, Morpholine and Dimethylpiperidine with a bromoacetic acid ester, respectively, to give the corresponding acetic acid ester derivatives, which were then deprotected to give the free carboxylic acids for activation. Detailed protocols are listed below:

Diisopropylamino-acetic acid-tert.butylester 2



2

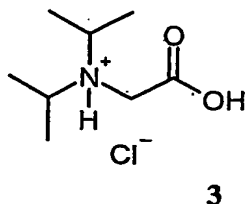
5.85 g (30 mMol) Bromoacetic acid-tert.butylester and 9.9 ml (70 mMol) Diisopropylamine were stirred for 5-7h under reflux in 50 ml THF. After removing the solvents, the product was dissolved in water and the pH of the solution was adjusted to pH 11 with 2N NaOH. The heterogeneous reaction mixture was extracted with EtOAc, washed with water and dried (sodium sulphate). The residue, after evaporation of the solvent was eluted from a silica gel column with dichloromethane/methanol (10/1 v/v).

yield: 5.4 g (84 %)

$R_F = 0.54$ ($\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH} = 5:1$)

$^1\text{H-NMR}$ (CDCl_3) δ 0.98 (d, 12H, iProp- CH_3), 1.42 (s, 9H, tert.Bu); 3.04 (m, 2H, iProp-CH); 3.07 (s, 2H, N- CH_2).

Diisopropylamino-acetic acid hydrochloride 3

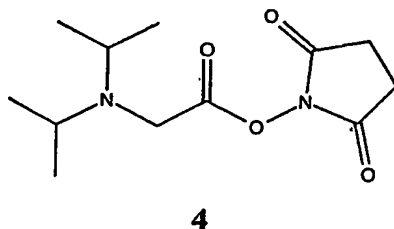


5.4 g (25 mMol) Diisopropylamino-acetic acid-tert.butylester 2 was stirred with 6.25 ml (75 mMol) 12 M HCl in 25 ml water for 2.5 h at 60°C. After distillation in vacuo of water and excess of HCl, the residue was dried with toluene azeotropically.

Yield: 4.1 g (84 %)

¹H-NMR (d₆-DMSO) δ 1.24 (d, 12H, iProp-CH₃); 3.66 (m, 2H, iProp-CH); 4.08 (s, 2H, N-CH₂); 8.6 (br.s, 2H, N⁺H, OH)

Diisopropylamino-acetic acid-(2,5-dioxo-pyrrolidine-1-yl)-ester 4



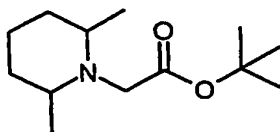
3.82 g (19.5 mMol) diisopropylamino-acetic acid hydrochloride (3), in 50 ml DMF, was added to 2.24 g (19.5 mMol) N-hydroxysuccinimide and 8.04 g (39 mMol) dicyclohexylcarbodiimide. The reaction mixture was stirred for 16h at RT. The residue was filtered and the filtrate was evaporated in vacuum to dryness. After dissolving the residue in CH₂Cl₂, the solution was washed with NaHCO₃, dried (sodium sulphate) and the solvent evaporated. The compound was then recrystallised from diisopropylether.

Yield: 3.5 g (70 %)

Mp. 96°C

$^1\text{H-NMR}$ (CDCl_3) δ 1.04 (d, 12H, iProp- CH_3), 2.81 (s, 4H, O-Su); 3.11 (m, 2H, iProp-CH); 3.59 (s, 2H, N- CH_2)

(2,6-Dimethyl-piperidine-1-yl)-acetic acid-tert.butylester 5



5

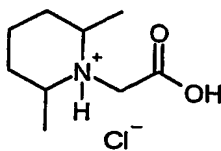
14.8 ml (100 mMol) Bromoacetic acid-tert.butylester 1 and 29.9 ml (230 mMol) 2,6-Dimethyl-piperidine were stirred for 5h at reflux in 150 ml THF. After removing the solvents, the product was dissolved in water and the pH of the solution was altered to pH 11 with 2N NaOH. The heterogeneous reaction mixture was extracted with EtOAc, washed with water and dried (sodium sulphate). The residue, after evaporation of the solvent, was eluted from a silica gel column with diisopropylether

Yield: 21,6 g (95 %)

R_F = 0,58 (Diisopropylether)

$^1\text{H-NMR}$ (CDCl_3) δ 1,09 (d, 6H, 2x N-C- CH_3), 1,16-1,67 (m, 6H, Pip- $\text{CH}_2\text{-CH}_2\text{-CH}_2$); 1,43 (s, 9H, tert.Bu); 2,81 (m, 2H, 2x N-CH); 3,45 (s, 2H, N- CH_2)

(2,6-Dimethyl-piperidine-1-yl)-acetic acid hydrochloride 6



6

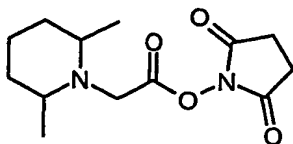
21.6 g (95 mMol) (2,6-Dimethyl-piperidine-1-yl)-acetic acid-tert.butylester (5) was stirred with 23.75 ml (285 mMol) 12 M HCl in 100 ml water for 2h at 60°C. After distillation in vacuo of water and excess of HCl, the residue was dried with toluene azeotropically.

Yield: 19.5 g (>98 %)

$R_F = 0.18$ ($\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH} = 5:1$)

$^1\text{H-NMR}$ (d_6 -DMSO) δ 1.1-1.38 (m, 6H); 1.4-1.9 (m, 6H); 3.35-3.65 (m, 2H); 4.05 (s, 2H); 9.05 (br.s, 1H); 10.85 (s, 1H)

(2,6-Dimethyl-piperidine-1-yl)-acetic acid-(2,5-dioxo-pyrrolidine-1-yl)-ester 7



7

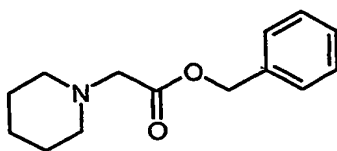
4.15 g (20 mMol) (2,6-Dimethyl-piperidin-1-yl)-acetic acid hydrochloride (6), in 50 ml DMF, was added to 2.3 g (20 mMol) N-hydroxysuccinimide and 8. g (40 mMol) Dicyclohexylcarbodiimide. The reaction mixture was stirred for 16h at RT. The residue was filtered and the filtrate was evaporated under vacuum to dryness. After dissolving the residue in CH_2Cl_2 , the solution was washed with NaHCO_3 , dried (sodium sulphate) and the solvent evaporated. The compound was then recrystallised from diisopropylether (A-Kohle).

Yield: 3.7 g (69 %)

Mp 72°C

$^1\text{H-NMR}$ (CDCl_3) δ 1.14 (d, 6H, 2x N-C-CH₃), 1.18-1.7 (m, 6H, Pip-CH₂-CH₂-CH₂); 2.75-2.85 (m, 2H, 2x N-CH); 2.82 (s, 4H, O-Su); 3.9 (s, 2H, N-CH₂)

(Piperidine-1-yl)-acetic acid-benzylester 9



9

11.45 g (50 mMol) Bromoacetic acid-benzylester 8 was carefully added to 9.36 g (110 mMol) Piperidine in 100 ml THF. The reaction mixture was stirred for 4h at reflux. After distillation of THF, the residue was solved in $\text{CH}_2\text{Cl}_2/\text{H}_2\text{O}$ and the pH of the solution was adjusted to pH10.5 with 2N NaOH. The product was then extracted in CH_2Cl_2 and dried over sodium sulphate. After evaporation of the solvent, the product was subjected to a chromatographic purification using ethyl acetate.

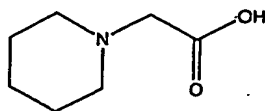
Yield: 10.4 g (89%)

R_F = 0.67 (EtOAc)

HPLC: >97% [ET 250/8/4 Nucleosil 7 μm Phenyl Machery & Nagel # 720019; Acetonitrile : (H_2O + 0,01M TBAS) = 95:5; flow 1 ml/Min.; 5,3 Min.]

$^1\text{H-NMR}$ (CDCl_3) δ 1,42 (m, 2H, C-C-CH₂-C-C); 1,57-1,62 (m, 4H, C-CH₂-C-CH₂-C); 2,49 (t, 4H, CH₂-N-CH₂); 3,21 (s, 2H, N-CH₂-CO); 5,14 (s, 2H, O-CH₂); 7,28-7,33 (m, 5H, Ph)

(Piperidine-1-yl)-acetic acid 10



10

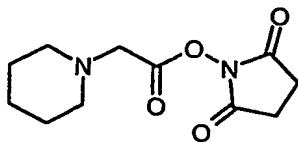
10.4 g (44 mMol) (Piperidine-1-yl)-acetic acid-benzylester 9 was stirred with 0.25 g Pd/C (5%) in 100 ml Methanol at RT. Under normal pressure, the reaction mixture was reduced (hydrated?) with H₂. After having removed the catalyst, the solution was evaporated and the residue was resuspended in EtOAc and filtered off.

Yield: 6 g (95%)

Mp 231°C

¹H-NMR (*d*₆-DMSO) δ 1.45 (m, 2H, C-C-CH₂-C-C); 1.62-1.67 (m, 4H, C-CH₂-C-CH₂-C); 2.89 (t, 4H, CH₂-N-CH₂); 3.19 (s, 2H, N-CH₂-CO); 4-6 (1H, OH)

(Piperidine-1-yl)-acetic acid-(2,5-dioxo-pyrrolidine-1-yl)-ester 11



11

6 g (42 mMol) (Piperidine-1-yl)-acetic acid 10 was added to 4.83 g (42 mMol) N-hydroxysuccinimide and 9.53 g (46.2 mMol) Dicyclohexyl-carbodiimide in 100 ml CH₂Cl₂. The reaction mixture was stirred for 16h at RT. The residue was filtered and the filtrate was evaporated in vacuum to dryness. The residue was then dissolved in Diethylether and the solution was filtered off. The filtrate was reduced once again and the residue was dissolved in

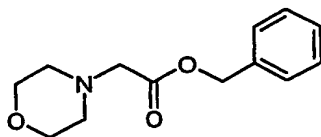
Diisopropylether. After filtration of the solution and evaporation of the solvent, the compound was then recrystallised from diisopropylether.

Yield: 7.1g (70%)

Mp: 82°C

$^1\text{H-NMR}$ (CDCl_3) δ 1.45 (m, 2H, C-C-CH₂-C-C); 1.58 (m, 4H, C-CH₂-C-CH₂-C); 2.6 (t, 4H, CH₂-N-CH₂); 2.84 (s, 4H, CO-CH₂-CH₂-CO); 3.54 (s, 2H, N-CH₂-CO)

(Morpholine-1-yl)-acetic acid-benzylester 12



12

11.45 g (50 mMol) Bromoacetic acid-benzylester 8 was carefully added to 9.58 g (110 mMol) Morpholine in 100 ml THF. The reaction mixture was stirred for 4h under reflux. After distillation of THF, the residue was solved in $\text{CH}_2\text{Cl}_2/\text{H}_2\text{O}$ and the pH of the solution was altered to pH10.5 with 2N NaOH. The product was then extracted in CH_2Cl_2 and dried over sodium sulphate. After evaporation of the solvent, the product was purified by chromatography using ethyl acetate.

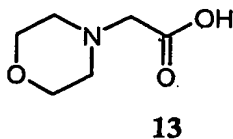
Yield: 11 g (93.5%)

R_F = 0.63 (EtOAc)

HPLC: >97% [ET 250/8/4 Nucleosil 7 μm Phenyl Machery & Nagel # 720019; Acetonitrile : (water + 0.01M TBAS) = 95:5; flow 1 ml/Min.; 4.0 Min.]

$^1\text{H-NMR}$ (CDCl_3) δ 2.56 (t, 4H, CH₂-N-CH₂); 3.23 (s, 2H, N-CH₂-CO); 3.72 (t, 4H, CH₂-O-CH₂); 5.14 (s, 2H, O-CH₂); 7.28-7.33 (m, 5H, Ph)

(Morpholin-1-yl)-acetic acid 13



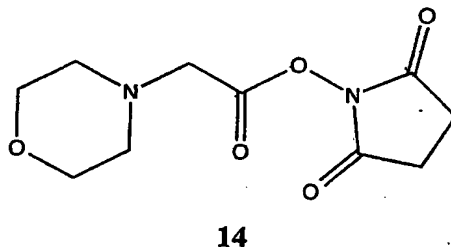
11 g (46 mMol) (Morpholin-1-yl)-acetic acid-benzylester 12 was stirred with 0.25 g Pd/C (5%) in 100 ml Methanol at RT. Under atmospheric pressure, the reaction mixture was hydrogenated with H₂. After having removed the catalyst, the solution was evaporated and the residue was resuspended in EtOAc and filtered off.

Yield: 6.3 g (95%)

Mp: 166°C

¹H-NMR (*d*₆-DMSO) δ 2.57 (t, 4H, CH₂-N-CH₂); 3.14 (s, 2H, N-CH₂-CO); 3.58 (t, 4H, CH₂-O-CH₂); 3.19; 9-12 (1H, OH)

(Morpholine-1-yl)-acetic acid-(2,5-dioxo-pyrrolidine-1-yl)-ester 14



6.3 g (44 mMol) (Morpholin-1-yl)-acetic acid 13 was added to 5.06 g (44 mMol) N-Hydroxysuccinimide and 9.98 g (48.4 mMol) Dicyclohexyl-carbodiimide in 100 ml CH₂Cl₂. The reaction mixture was stirred for 16h at RT. The residue was filtered and the filtrate was evaporated in vacuum to dryness. The residue was then dissolved in Diethylether and the solution was filtered off. The filtrate was reduced once again and the residue was dissolved in

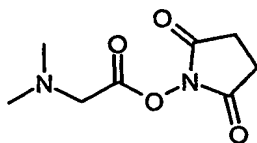
Diisopropylether. After filtration of the solution and evaporation of the solvent, the compound was then recrystallized from diisopropylether.

Yield: 8.1g (76%)

Mp: 142°C

$^1\text{H-NMR}$ (CDCl_3) δ 2.62 (t, 4H, $\text{CH}_2\text{-N-CH}_2$); 2.8 (s, 4H, $\text{CO-CH}_2\text{-CH}_2\text{-CO}$); 3.52 (s, 2H, $\text{N-CH}_2\text{-CO}$); 3.69 (t, 4H, $\text{CH}_2\text{-O-CH}_2$)

Dimethylamino-acetic acid-(2,5-dioxo-pyrrolidine-1-yl)-ester 16



16

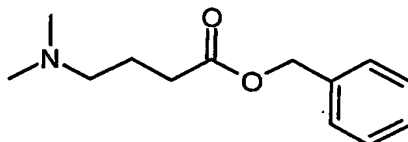
5.15 g (50 mMol) Dimethylamino-acetic acid 15 was added to 5.75 g (50 mMol) N-Hydroxy-succinimide and 11.05 g (55 mMol) Dicyclohexyl-carbodiimide in 100 ml CH_2Cl_2 at 0°C. The reaction mixture was stirred for 20h at RT. The residue was filtered and the filtrate was evaporated in vacuum to dryness. The residue was then dissolved in Diethylether, mixed with A-Kohle. The solution was filtered off and evaporated. The obtained oil was dissolved in a small amount of Diethylether to get a crystallization of the product. The cooled crystal solution was filtered off. The crystals were once again dissolved in Diethylether, treated with A-Kohle and the solution was filtered off. Those purification steps were repeated several times until the obtaining of a pure product in $^1\text{H-NMR}$

Yield: 0.5-1 g (5-10 %)

Mp: 67°C

$^1\text{H-NMR}$ (CDCl_3) δ 2.42 (s, 6H, 2x CH_3); 2.82 (s, 4H, O-Su); 3.52 (s, 2H, N-CH_2)

4-Dimethylamino-butyric acid-benzylester 19



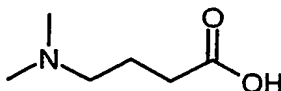
19

25 g (150 mMol) 4-Dimethylamino-butyric acid hydrochloride 18, 27,4g (254 mMol) Benzylalcohol und 29.5 g (155 mMol) 4-Toluenesulphonic acid-Monohydrate were stirred in 200 ml Toluene at reflux and water was removed by a water separator for ca. 2h. After cooling the reaction mixture, the toluene phase was extracted 4 times with water. The aqueous phase was then extracted 3 times with EtOAc and the pH of the solution was adjusted to pH 9.5 with NaOH. The product was extracted with ethylacetate. The organic layer was washed with saturated NaCl-solution, dried and evaporated to afford the pure Benzylester.

Yield: 30.4 g (92 %)

$^1\text{H-NMR}$ (CDCl_3) δ 1.8 (m, 2H, N-C-CH₂); 2.17 (s, 6H, 2x CH₃); 2.26 (t, 2H, CH₂-CO); 2.38 (t, 2H, N-CH₂); 5.11 (s, 2H, O-CH₂); 7.28-7.37 (m, 5H, Ph)

4-Dimethylamino-butyric acid 20



20

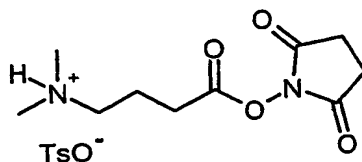
85 g (380 mMol) 4-Dimethylamino-butyric acid-benzylester 19 was stirred with 1 g Pd/C (5%) in 500 ml Methanol. The reaction mixture was treated with hydrogen for 4h. After removing the catalyst, methanol was distilled. A small volume of ethylacetate was added to the remaining

residue so that the product would crystallise. The product was then filtered off and washed with ethylacetate.

Yield: 42.5 g (85 %)

$^1\text{H-NMR}$ (d_6 -DMSO) δ 1.63 (m, 2H, N-C-CH₂); 2.22 (s, 6H, 2x CH₃); 2.21 (t, 2H, CH₂-CO); 2.37 (t, 2H, N-CH₂); 5.8 (br.s, 1H, OH)

3-Dimethylamino-butyric acid-(2,5-dioxo-pyrrolidine-1-yl)-ester hydrogentosylate 22



22

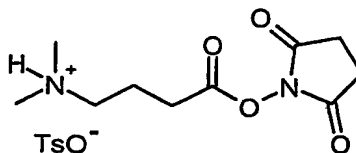
6.55 g (50 mMol) 3-Dimethylamino-butyric acid **20** was added to 5.75 g (50 mMol) N-Hydroxysuccinimide and 11.05 g (55 mMol) Dicyclohexyl-carbodiimide in 100 ml CH₂Cl₂. The reaction mixture was stirred for 16h at RT. The obtained urea was filtrated and the solution was evaporated. The residue, dissolved in 10 ml Acetonitrile, was added under stirring to a solution of 9.51 g (50 mMol) 4-Toluenesulfonic acid monohydrate in Acetonitrile (30 ml). The solvent was evaporated under vacuum and the product was recrystallized from Acetonitrile or Acetone.

Yield: 13.8 g (69%)

Mp 133°C

$^1\text{H-NMR}$ (d_6 -DMSO) δ 1.97 (m, 2H, CH₂-CO); 2.29 (s, 3H, H₃C-Ph), 2.76-2.82 ("m", 12H, 2x CH₃, O-Su, C-CH₂-C); 3.12 (m, 2H, N-CH₂); 7.13 and 7.50 (m, m, 4H, Ph); 9.38 ("s"; 1H, N⁺H)

3-Dimethylaminobutyric acid-(2,5-dioxo-pyrrolidin-1-yl)-ester hydrogentosylate 22



22

2.62 g (20 mMol) 3-Dimethylamino-butyric acid 20 was added to 2.3 g (20 mMol) N-Hydroxysuccinimide and 3.8 g (20 mMol) p-Toluenesulfonic acid monohydrate and 8.25 g (40 mMol) Dicyclohexylcarbodiimide in 100 ml Acetonitrile. After stirring the reaction mixture for 16h at RT, the obtained urea compound was removed and the filtrate evaporated. The residue was recrystallized from Acetone.

Yield: 7 g (86%)

Mp 133°C

¹H-NMR (*d*₆-DMSO) δ 1.97 (m, 2H, CH₂-CO); 2.29 (s, 3H, H₃C-Ph), 2.76-2.82 ("m", 12H, 2x CH₃, O-Su, C-CH₂-C); 3.12 (m, 2H, N-CH₂); 7.13 and 7.50 (m, m, 4H, Ph); 9.38 ("s"; 1H, N⁺H)

Coupling of Basic Mass Tags to beta-MSH

30nmol BMSH was dissolved in 30mM BUT (BUT: Buffer of Borate Urea Thiourea), pH7.5. 30mM BMT was then added to the peptide solution. After stirring for 2h, the same amount of BMT was added again and stirred for 2h. The reaction mixture was then purified with a SCX cartridge to remove the excess of BMTs and the peptide mixture was analysed in LC-MS (LCQ).

Labelling of a test protein with a Basic Mass Tag

A protocol that is generally applicable to biological samples was used. 500 μ g of a commercially available protein, Rabbit Pyruvate Kinase, was first digested with trypsin; 30 μ g trypsin (seq. grade modified trypsin, Promega) were added, the pH was adjusted to 7.8 and the solution was incubated at 37°C for 24 hours. The polypeptide was then dissolved in a BUT buffer (final concentration: Borate 30 mM, 1.5 M urea, 0.7 M thiourea) and reduced with TCEP (1 mM) for

1 h at RT, pH 7.5. After labelling the reduced thiols with iodoacetamide (5 mM), a solution of DMG N-hydroxysuccinimide ester (30 mM final concentration) was added to the reaction mixture and stirred for 4 hours at Room Temperature. The same amount of tag was added a second time and the reaction mixture was stirred at least 4 more hours. To remove the excess of labelling reagents, the guanidine hydrochloride and the DMF and to prepare the sample for MS analysis, the labelled polypeptides sample was purified by an extraction cartridge (HLB Oasis, Waters Corp., Milford, Mass USA).

MS and MS/MS analysis

Analyses were performed by liquid chromatography mass spectrometry using a Finnigan LCQ Deca with a Finnigan Surveyor HPLC System (Column: Thermo-Hypersil-Keystone BioBasic, 100 x 1 mm, 5µm HyPURITY™ Elite C18; Solvents: 100% Water to 100 % Methanol both with 0.2% Formic Acid).

Peptide identification was performed by CID (collision of peptide ions selected using 'dynamic exclusion' followed by automatic interpretation of the MS/MS spectra by SEQUEST (Yates, J. R., 3rd; Eng, J. K.; McCormack, A. L., Anal Chem. 67(18) pages 3202 – 3210, "Mining genomes: correlating tandem mass spectra of modified and unmodified peptides to sequences in nucleotide databases", 1995; MacCoss, M. J.; Wu, C. C.; Yates, J. R., 3rd, Anal Chem. 74(21) pages 5593 – 5599, "Probability-based validation of protein identifications using a modified SEQUEST algorithm", 2002).

Demonstration of Small and Predictable Mobility Shift in Peptides Labelled with DMG

The peptides from the tryptic digest of Rabbit Pyruvate Kinase labelled with the N-hydroxysuccinimide active ester of dimethylglycine (DMG) prepared as described was analysed by HPLC-MS/MS with Sequest to identify peptides in parallel with the corresponding analysis of the tryptic digest from the unlabelled Rabbit Pyruvate Kinase. The retention times of singly labelled and unlabelled peptides that were identified in both analyses were recorded and the retention times were plotted against each other in figure 2.

Linear regression analysis was performed on this data. When parameters were estimated for the regression it was found that the least squares error was lowest for the intercept that corresponded to a 1.67 minute delay in retention time for a single tag on a peptide when compared with the retention time for the unlabelled peptide. The solid line in figure 2 marks where the points would fall if the retention time was the same for labelled and unlabelled peptides, while the broken line marks the regression line. The R^2 value for this regression was 0.97 suggesting that it is an accurate model of the effect of the DMG tag on singly labelled peptides.

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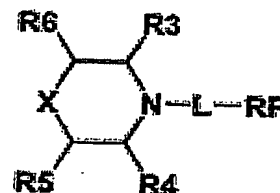
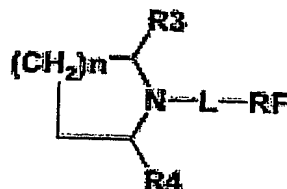
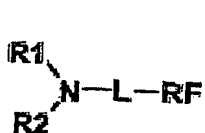
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CLAIMS:

1. A method for characterising a molecule by mass spectrometry, which molecule comprises one or more free amino groups, which method comprises:
 - (a) reacting one or more free amino groups in the molecule with a mass tag reagent comprising a reactive functionality capable of reacting with an amino group, and a tertiary amino group linked to the reactive functionality; and
 - (b) characterising the molecule by mass spectrometry.
2. A method according to claim 1, wherein the reactive functionality, is selected from active esters of carbonic acids, alkenyl sulphones, haloalkanes, maleimides, isocyanates, isothiocyanates, ketones, aldehydes, sulphonyl-halides, carboxylic-halides, anhydride esters and alkenes.
3. A method according to claim 1 or claim 2, wherein the tertiary amino group comprises a dialkyl substituted amino group, in which the alkyl groups may together with the nitrogen to which they are attached, form a cyclic group, and wherein the cyclic group may comprise an N, S and/or O atom.
4. A method according to any preceding claim, wherein the tertiary amino group is linked to the reactive functionality by a linker selected from an alkylene group and a phenylene group.
5. A method according to any preceding claim, wherein where the mass tag reagent is selected from a compound having one of the following formulae:



wherein R^1 and R^2 may be the same or different and are independently selected from alkyl groups and aralkyl groups;

R^3 , R^4 , R^5 and R^6 may be the same or different and are independently selected from hydrogen and alkyl groups;

n is an integer of 0, 1 or 2;

X is selected from N-alkyl, S and O;

the reactive functionality RF is selected from active esters of carbonic acids, alkenyl sulphones, haloalkanes, maleimides, isocyanates, isothiocyanates, ketones, aldehydes, sulphonyl-halides, carboxylic-halides, anhydride esters, alkenes, N-hydroxysuccinimide esters, hydroxybenzotriazole esters, hydroxyazabenzotriazole esters, nitrophenyl esters, trichlorophenyl esters and pentafluorophenyl esters; and

the linker L is selected from an alkylene group and a phenylene group.

6. A method according to claim 5, wherein R^1 and R^2 are independently selected from methyl, ethyl, propyl, iso-propyl, cyclohexyl, benzyl, and substituted benzyl groups.
7. A method according to claim 5 or claim 6, wherein R^3 , R^4 , R^5 and R^6 are independently selected from hydrogen and methyl, ethyl, propyl, and iso-propyl groups.
8. A method according to any of claims 5-7, wherein n is 2.

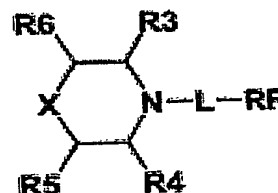
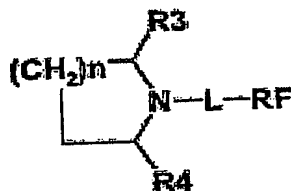
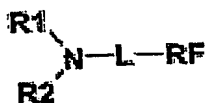
9. A method according to any of claims 5-8, wherein X is O;
10. A method according to any of claims 5-8, wherein when X is N-alkyl, alkyl is selected from a methyl, ethyl, propyl, iso-propyl or cyclohexyl group.
11. A method according to any of claims 5-10, wherein RF is selected from esters of carbonic acid, N-hydroxysuccinimide esters, hydroxybenzotriazole esters, hydroxyazabenzotriazole esters, nitrophenyl esters, trichlorophenyl esters and pentafluorophenyl esters.
12. A method according to any of claims 5-11, wherein L is selected from substituted or unsubstituted $-(CH_2)_n-$ in which n is an integer of from 1 to 5, and substituted or unsubstituted $-(C_6H_4)-$.
13. A method according to claim 12, wherein L is selected from $-CH_2-$ and $-(CH_2)_3-$.
14. A method according to any preceding claim, wherein the mass tag reagent is an ester of dimethylaminoglycine.
15. A method according to any preceding claim, wherein the mass tag reagent has a modified isotope distribution.
16. A method according to claim 15, wherein the isotope distribution of one or more of the following types of atoms is modified in the mass tag reagent: hydrogen, carbon, nitrogen, oxygen, sulphur and a halogen.
17. A method according to claim 16, wherein the isotope distribution is modified by replacing a portion of 1H by 2H , a portion of ^{12}C by ^{13}C , a portion of ^{16}O by ^{18}O and/or a portion of ^{14}N by ^{15}N .

18. A method according to any preceding claim, wherein the molecule comprises a protein, a polypeptide, a peptide or an amino acid, or a derivative of the above.
19. A method according to claim 18, which method comprises:
- (a) labelling free amino functionalities in the molecule with a mass tag reagent;
 - (b) cleaving the molecule with a sequence specific cleavage reagent, to produce cleavage products;
 - (c) labelling the free amino groups generated in the molecule by the cleavage reagent with a second mass tag reagent; and
 - (d) analysing the resulting cleavage products by mass spectrometry.
20. A mass tag reagent for labelling a molecule to be characterised by mass spectrometry, which mass tag reagent has the following formula:

tertiary amino group-L-reactive functionality

wherein the reactive functionality, is selected from esters of carbonic acids, alkenyl sulphones, haloalkanes, maleimides, isocyanates, isothiocyanates, ketones, aldehydes, sulphonyl-halides, carboxylic-halides, anhydride esters, alkenes, N-hydroxysuccinimide esters, hydroxybenzotriazole esters, hydroxyazabenzotriazole esters, nitrophenyl esters, trichlorophenyl esters and pentafluorophenyl esters; and wherein the tertiary amino group comprises a dialkyl substituted amino group, in which the alkyl groups may together with the nitrogen to which they are attached, form a cyclic group, and wherein the cyclic group may comprise an N, S and/or O atom; and wherein L selected from an alkylene group and a phenylene group.

21. A mass tag reagent according to claim 20, having one of the following formulae:



wherein R^1 and R^2 may be the same or different and are independently selected from alkyl groups and aralkyl groups;

R^3 , R^4 , R^5 and R^6 may be the same or different and are independently selected from hydrogen and alkyl groups;

n is an integer of 0, 1 or 2;

X is selected from N-alkyl, S and O;

the reactive functionality RF is selected from active esters of carbonic acids, alkenyl sulphones, haloalkanes, maleimides, isocyanates, isothiocyanates, ketones, aldehydes, sulphonyl-halides, carboxylic-halides, anhydride esters, alkenes, N-hydroxysuccinimide esters, hydroxybenzotriazole esters, hydroxyazabenzotriazole esters, nitrophenyl esters, trichlorophenyl esters and pentafluorophenyl esters; and

the linker L is selected from an alkylene group and a phenylene group.

22. A mass tag reagent according to claim 20 or claim 21, wherein R^1 and R^2 are independently selected from methyl, ethyl, propyl, iso-propyl, cyclohexyl, benzyl, and substituted benzyl groups.

23. A mass tag reagent according to any of claims 20-22, wherein R^3 , R^4 , R^5 and R^6 are independently selected from hydrogen and methyl, ethyl, propyl, and iso-propyl groups.

24. A mass tag reagent according to any of claims 20-23, wherein n is 2.
25. A mass tag reagent according to any of claims 20-24, wherein X is O;
26. A mass tag reagent according to any of claims 20-24, wherein when X is N-alkyl, alkyl is selected from a methyl, ethyl, propyl, iso-propyl or cyclohexyl group.
27. A mass tag reagent according to any of claims 20-26, wherein RF is selected from esters of carbonic acid, N-hydroxysuccinimide esters, hydroxybenzotriazole esters, hydroxyazabenzotriazole esters, nitrophenyl esters, trichlorophenyl esters and pentafluorophenyl esters.
28. A mass tag reagent according to any of claims 20-27, wherein L is selected from substituted or unsubstituted $-(CH_2)_n-$ in which n is an integer of from 1 to 5, and substituted or unsubstituted $-(C_6H_4)-$.
29. A mass tag reagent according to any of claims 20-28, wherein L is selected from $-CH_2-$ and $-(CH_2)_3-$.
30. A mass tag reagent according to any of claims 20-29, wherein the mass tag reagent has a modified isotope distribution.
31. A mass tag reagent according to any of claims 20-30, wherein the isotope distribution of one or more of the following types of atoms is modified in the mass tag reagent: hydrogen, carbon, nitrogen, oxygen, sulphur and a halogen.

32. A mass tag according to claim 31, wherein the isotope distribution is modified by replacing a portion of ^1H by ^2H , a portion of ^{12}C by ^{13}C , a portion of ^{16}O by ^{18}O and/or a portion of ^{14}N by ^{15}N .
33. An array of mass tags for labelling one or more molecules to be characterised by mass spectrometry, which array comprises two or more mass tags as defined in any of claims 19-32.
34. An array of mass tags according to claim 33, wherein every mass tag in the array has the same chemical structure, and each mass tag in the array is an isotopomer of the other mass tags in the array such that each mass tag in the array has a different mass.
35. A kit for purification of labelled analyte molecules, which kit comprises one or more mass tags as defined in any of claims 20-34, and a cation exchange resin.
36. A kit according to claim 35, which kit further comprises one or more components selected from a reaction buffer for the coupling of the tag to analyte molecules, a buffer for washing the cation exchange resin, and a buffer for elution of the labelled peptides from the cation exchange resin.

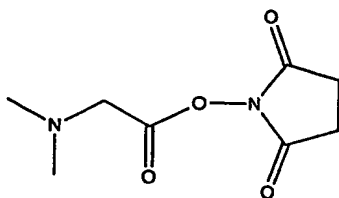
ABSTRACT

MASS LABELS

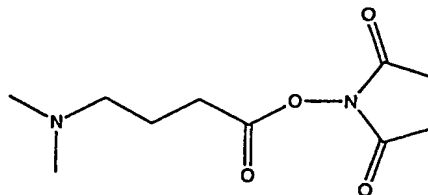
Provided is a method for characterising a molecule by mass spectrometry, which molecule comprises one or more free amino groups, which method comprises:

- (a) reacting one or more free amino groups in the molecule with a mass tag reagent comprising a reactive functionality capable of reacting with an amino group, and a tertiary amino group linked to the reactive functionality; and
- (b) characterising the molecule by mass spectrometry.

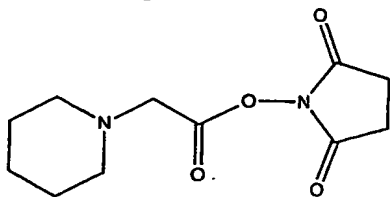
FIGURE 1



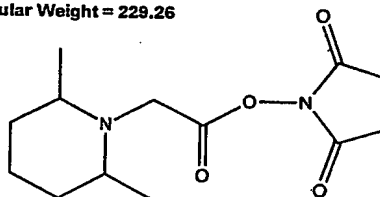
Dimethylamino-acetic acid N-hydroxysuccinimide ester
Molecular Weight = 200.20



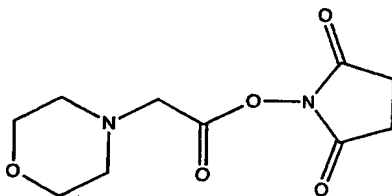
Toluene-4-sulfonate Dimethylamino-butyric acid
N-hydroxysuccinimide ester
Molecular Weight = 229.26



Piperidin-1-yl-acetic acid N-hydroxysuccinimide ester
Molecular Weight = 240.26



2,6-Dimethyl-Piperidin-1-yl-acetic acid N-hydroxysuccinimide ester
Molecular Weight = 268.32



Morpholino-4-yl-acetic acid N-hydroxysuccinimide ester
Molecular Weight = 242.23

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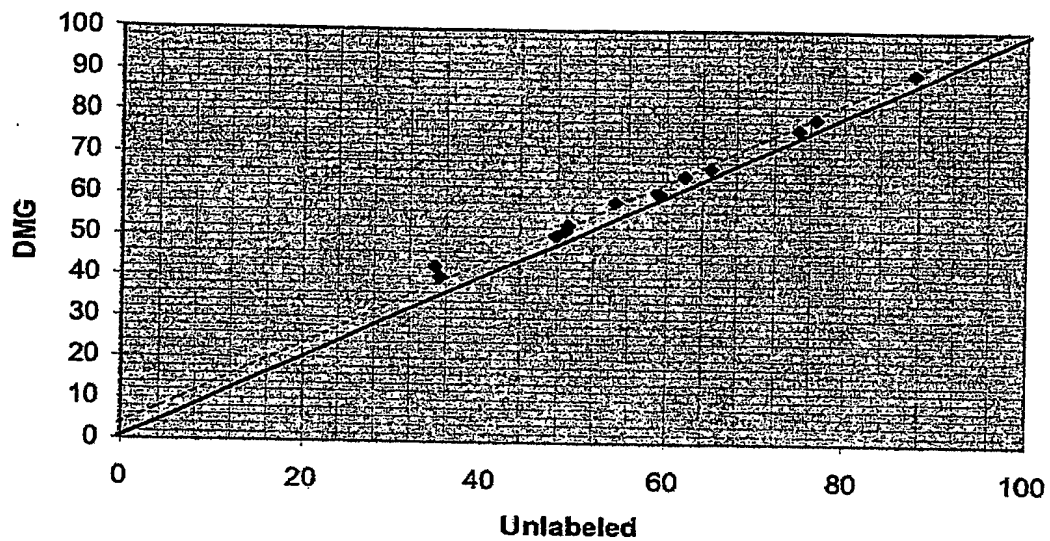
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FIGURE 2



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